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DOI:

[10.1016/j.pupt.2017.10.008](https://doi.org/10.1016/j.pupt.2017.10.008)

*Document Version*

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*Citation for published version (APA):*

Riffo-Vasquez, Y., Venkatasamy, R., & Page, C. P. (2018). Steroid sparing effects of doxofylline. *Pulmonary pharmacology & therapeutics*. <https://doi.org/10.1016/j.pupt.2017.10.008>

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# Accepted Manuscript

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Yanira Riffo-Vasquez, Radhakrishnan Venkatasamy, Clive P. Page

PII: S1094-5539(17)30221-3

DOI: [10.1016/j.pupt.2017.10.008](https://doi.org/10.1016/j.pupt.2017.10.008)

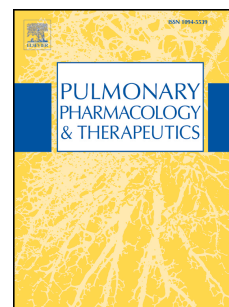
Reference: YPUPT 1672

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 12 September 2017

Revised Date: 11 October 2017

Accepted Date: 12 October 2017



Please cite this article as: Riffo-Vasquez Y, Venkatasamy R, Page CP, Steroid sparing effects of doxofylline, *Pulmonary Pharmacology & Therapeutics* (2017), doi: 10.1016/j.pupt.2017.10.008.

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**STEROID SPARING EFFECTS OF DOXOFYLLINE**

Yanira Riffo-Vasquez, PhD, Radhakrishnan Venkatasamy, PhD, Clive P. Page, PhD

Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, UK.

\*Corresponding author: Yanira Riffo-Vasquez, PhD, Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, 150 Stamford Street, London SE1 9NH, UK. Tel no: +44 207 8484819, FAX no +44 207 8486097. Email: yanira.vasquez@kcl.ac.uk

Word count: 1,818

Key Words: Doxofylline, Steroid Sparing, Lung, Inflammation

## ABSTRACT

Glucocorticosteroids are widely used in the treatment of asthma and chronic obstructive pulmonary disease (COPD). However, there are growing concerns about the side effect profile of this class of drug, particularly an increased risk of pneumonia. Over the last two decades there have been many attempts to find drugs to allow a reduction of glucocorticosteroids, including xanthines such as theophylline. Use of xanthines has been shown to lead to a reduction in the requirement for glucocorticosteroids, although xanthines also have a narrow therapeutic window limiting their wider use. Doxofylline is another xanthine that has been shown to be of clinical benefit in patients with asthma or COPD, but to have a wider therapeutic window than theophylline. In the present study we have demonstrated that doxofylline produces a clear steroid sparing effect in both an allergic and a non-allergic model of lung inflammation. Thus, we have shown that concomitant treatment with a low dose of doxofylline and a low dose of the glucocorticosteroid dexamethasone (that alone had no effect) significantly reduced both allergen-induced eosinophil infiltration into the lungs of allergic mice, and lipopolysaccharide (LPS)-induced neutrophil infiltration into the lung, equivalent to a higher dose of each drug. Our results suggest that doxofylline demonstrates significant anti-inflammatory activity in the lung which can result in significant steroid sparing activity.

## INTRODUCTION

Glucocorticosteroids are widely used drugs in the treatment of patients with asthma or COPD[1]. However, this class of drug are known to exhibit a wide variety of side effects which has prompted considerable efforts to find pharmacological approaches to try and reduce the dose of glucocorticosteroids without compromising efficacy. Thus, recent clinical data has shown that treatment with anti-IL5 can reduce glucocorticosteroid usage in patients with severe asthma [2] and the addition of a long acting B2 agonist to a low dose of an inhaled glucocorticosteroid has been demonstrated to produce an improved clinical outcome to doubling the dose of the inhaled glucocorticosteroid suggested as the scientific justification for the success of combination inhalers [3]. Xanthines are drugs widely used in the treatment of respiratory diseases and exhibit both bronchodilator and anti-inflammatory actions [4]. However, xanthines such as theophylline have a very narrow therapeutic window and elicit a wide range of dose-dependent side effects [5, 6]. Furthermore, xanthines must be used with caution when co-prescribed with other drugs as there are many reported drug/drug interactions that can also lead to adverse effects. This has led to the search for newer drugs with an improved safety profile, including enprofylline, bamifylline and doxofylline which have been referred to as “novofyllines” [7]. Clinical data has suggested that doxofylline has a wider therapeutic window than theophylline, and exhibits less cardiovascular and gastrointestinal side effects (Reviewed in [7]). Recent experimental work has suggested that this improved therapeutic window may be due to doxofylline lacking either significant adenosine receptor antagonism or inhibition of phosphodiesterase enzymes (PDEs), such as PDE3 expressed in the cardiovascular system [8], mechanisms that are thought to contribute to the side effect profile of theophylline [9].

We, and others, have demonstrated that doxofylline has significant anti-inflammatory actions [10, 11], including the ability of this drug to reduce leukocyte recruitment into the airways [12]. However, whilst there have been a number of studies reporting the ability of theophylline to have a steroid sparing action [13, 14], and indeed to have complimentary anti-inflammatory activity to corticosteroids, there is only limited data with doxofylline. Given the recent concern about the increased risk of pneumonia in patients with asthma [15] or COPD [16], regularly prescribed corticosteroids, and in the recognition that this class of drugs have only limited benefit in many patients with COPD, there have been many new pharmacological approaches to reduce the need for corticosteroids in patients with respiratory diseases. Since doxofylline has been demonstrated to have anti-inflammatory activity [12] and to show significant clinical benefit in both adults and children with asthma or COPD, we have investigated whether doxofylline can exhibit corticosteroid sparing activity in murine models of non-allergic (LPS) and allergen-induced leukocyte infiltration into the lung. We have utilised bacterial LPS as the model for non allergic leukocyte infiltration as this is known to be a good stimulus to induce neutrophil recruitment into the lung, both experimentally [17, 18] and clinically [19-21], and we have used a well established model of allergic inflammation in the airways to investigate whether doxofylline can have corticosteroid sparing activity against allergen-induced eosinophil infiltration into the lungs of mice.

## METHODS

### Animals

Male and female BALB/c mice, 6 to 8 weeks old, were used in this study (Envigo, UK). Experiments were approved by the Home Office under The Animals (Scientific Procedures) Act (1986) and local approval from the Ethics Committee of King's College London. Our research was carried out adhering to the recommendations of the ARRIVE guidelines for using animals in research [22]

### Non allergic Inflammation

Male Balb/c mice received 10 µg of LPS (*E. Coli*, Sigma, UK) intranasally under light anaesthesia with isoflurane.

### Allergic Inflammation

Female Balb/c mice were immunized intra-peritoneally with 30 µg of chicken egg albumin (OVA type V; Sigma, UK) absorbed to a saturated solution of aluminium hydroxide (2.5 mg/ml; Sanofi, Brazil) [23, 24] [23, 24] [23, 24]. Controls received aluminium hydroxide only. Five and 10 days later (day 5 and 10) the injection of OVA was repeated. On day 14 to 16, all animals were challenged with an aerosolised solution of OVA (3%) for 25 minutes, once daily.

### Drug treatment

Doxofylline (Eurodrug Laboratories, The Hague, The Netherlands) was administered intraperitoneally at 0.1, or 1mg/kg -24, -1 before, and 6h after intra-nasal instillation of 10µg of LPS or aerosol challenge with OVA. Dexamethasone (Sigma, UK) was injected intraperitoneally at 0.1, or 1 mg/kg -24h and 10 minutes before intra-nasal instillation of 10µg of LPS or aerosol challenge with OVA. Montelukast Sodium (Pharmacopeia reference Standard) was injected intraperitoneally at 0.1 or 0.3 mg/kg -24h and 10 minutes before

aerosol challenge with OVA. Control mice were treated with saline only. In some experiments we administered a low dose of doxofylline (0.1 mg/kg) or montelukast (0.1 mg/kg) with a low dose of dexamethasone (0.1 mg/kg).

### **Bronchoalveolar lavage**

Twenty-four hours after LPS instillation, or the last OVA challenge in allergic animals, mice were euthanized with an overdose of urethane (25% solution i.p.; Sigma Chemical Co.) and a cannula was inserted into the exposed trachea and three 0.5 ml aliquots of saline were injected into the lungs. From the BAL fluid, an aliquot (50  $\mu$ l) was added to 50  $\mu$ l of haemolysis solution (Turk's solution, Fluka, UK). The total number of cells in the lavage was counted with an improved Neubauer haemocytometer. For differential cell counts, cytopspin preparations were prepared from aliquots of BAL fluid (100  $\mu$ L) centrifuged at 1000 rpm for 1 min using a Shandon Cytospin 2 (Shandon Southern Instruments, Sewickley, PA, USA) at room temperature. Cells were stained with Diff Quick (DADE Behring, Germany) and a total of 100 cells were counted to determine the proportion of neutrophils, eosinophils and monocytes using standard morphological criteria.

### **Statistical analysis**

Data were analyzed using ANOVA followed where appropriate by a post hoc test (Bonferroni; SPSS version 20) and differences between mean values considered significant if  $p < 0.05$ .



## RESULTS

### LPS-induced neutrophil migration into the lung lumen

The total number of cells quantified in BAL fluid obtained from LPS- treated mice (LPS) 24 h following intra-nasal instillation was significantly higher compared to saline-treated mice (LPS:  $196.8 \pm 10.3 \times 10^4$  cells/ml, n=5 *versus* Saline:  $27.2 \pm 3.2 \times 10^4$  cells/ml; n=5;  $p < 0.05$ ). This was reflected by a significantly greater number of neutrophils recruited to the airways in LPS-treated mice ( $170.4 \pm 8.7 \times 10^4$  cells/ml) *versus* saline-treated mice ( $0.2 \pm 0.1 \times 10^4$  cells/ml;  $p < 0.0001$ , Figure 1A). The intensity of the inflammatory response was significantly reduced in animals treated with 1mg/Kg of dexamethasone ( $p < 0.0001$ , n=5 and 1mg/kg of doxofylline ( $p < 0.01$ , n=6), but not with the lower doses of either drug (0.1mg/kg). However, the combination of 0.1 mg/kg of dexamethasone and 0.1 mg/kg doxofylline, concentrations that alone did not significantly alter the response to LPS, did significantly inhibit the migration of neutrophils to the lung in response to 10  $\mu$ g of LPS (Fig. 1 B,  $p < 0.0001$ , n=5).

### OVA-induced eosinophil migration to the lung lumen

The total number of cells quantified in BAL fluid obtained from OVA-sensitized mice (OVA) 24 h following the last OVA challenge was significantly higher compared to sham-immunized mice (OVA:  $33.5 \pm 3.5 \times 10^4$  cells/ml, n=10 *versus* Saline:  $11.8 \pm 1 \times 10^4$  cells/ml; n=10,  $p < 0.001$ ). This was reflected by a significantly greater number of eosinophils recruited to the airways in OVA-sensitized mice ( $17.4 \pm 2.9 \times 10^4$  cells/ml  $p < 0.001$ , Figure 2A). The intensity of the inflammatory response was significantly reduced in animals treated with 1mg/kg, but not with 0.1 mg/kg of dexamethasone ( $p < 0.01$ , n=5), while with 1mg/kg of doxofylline again significantly reduced the inflammatory response ( $P < 0.01$ , n=5). However,

treatment with Montelukast (0.1 or 0.3 mg/kg) or doxofylline (0.1 mg/kg) alone did not significantly alter allergen-induced eosinophil infiltration (Figure 1). Nonetheless, the combination of 0.1 mg/kg of dexamethasone and 0.1 mg/kg doxofylline significantly inhibited the migration of eosinophils into the lung in response to OVA (Fig. 2 B,  $p < 0.0001$ ,  $n=6$ ). In contrast the combination of 0.1 mg/kg of dexamethasone and 0.1 mg/kg of montelukast did not significantly alter the response to OVA (Fig. 2 B,  $n=5$ ).

## DISCUSSION

We report here that doxofylline, a xanthine drug that has been used as a treatment for respiratory diseases for more than 30 years [7], is able to exhibit corticosteroid sparing activity in two murine models of lung inflammation. We have demonstrated that doxofylline produced a dose dependent inhibition of both LPS-induced lung neutrophilia in healthy animals as we have previously reported [12] and we have also demonstrated that this drug is able to inhibit allergen-induced eosinophil infiltration into the lungs in allergic mice. These results extend previous work demonstrating that doxofylline has anti-inflammatory activity of relevance to lung diseases such as asthma and COPD [12, 25, 26]. We have also demonstrated that the corticosteroid dexamethasone was able to inhibit both of these inflammatory insults as was to be expected from the wealth of data describing the anti-inflammatory effect of this class of drugs. However, the combination of doxofylline with dexamethasone at doses that themselves did not induce any significant reduction in the inflammation induced by LPS or allergen produced highly significant reductions in leukocyte infiltration into the lung in both models. Indeed the anti-inflammatory effect of the low dose dexamethasone in the presence of a low dose of doxofylline was equivalent to around a 10 times higher dose of dexamethasone administered alone. Clearly these results support the use of doxofylline as another drug able to reduce the need for corticosteroids, an observation that has also been recently observed in the clinic [27]. Our work lends further support to the considerable data in the literature showing that other xanthines also have corticosteroid sparing activity [14, 28].

Theophylline has been suggested to act co-operatively with corticosteroids by virtue of being able to interact with certain HDAC enzymes and thus enhancing the effect of corticosteroids on gene transcription in inflammatory cells, reducing the synthesis of pro-inflammatory mediators [13]. However, in previous work we found no evidence for doxofylline sharing this

effect with theophylline on any of the known HDAC enzymes [8] suggesting that the observed corticosteroid sparing effect of doxofylline in the present work is unlikely to be via an HDAC mediated mechanism. Furthermore, whilst xanthines are sometimes classified as non selective PDE inhibitors, and that particularly inhibition of PDE4 is known to be associated with anti-inflammatory activity, our previous work has suggested this is also not a property exhibited by doxofylline [8]. Thus, the precise mechanism of action of doxofylline to explain this corticosteroid sparing effect remains unknown. Nonetheless, we believe our observations are of considerable interest as they provide a good scientific rationale to further investigate doxofylline as a corticosteroid sparing agent in the clinic given it is a safe, orally active and effective drug for the treatment of asthma and COPD (6). Indeed a recent clinical study has suggested a corticosteroid sparing effect of doxofylline [29] which is supported by recent pharmacoeconomic data from Italy that in patients prescribed doxofylline for the treatment of their respiratory disease, there is less use of corticosteroids than in patients prescribed theophylline [30].

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## Legend of the figures

**Figure 1. (A)** Effect of dexamethasone or doxofylline on LPS-induced neutrophil migration to the lung. **(B)** Effect of the combination of dexamethasone/doxofylline on LPS-induced neutrophil migration to the lung. Bronchoalveolar lavage was collected 24 h post saline instillation (Saline) or instillation of 10 µg of LPS i.n. (LPS). Mice were treated with doxofylline (0.1 or 1mg/Kg i.p.), dexamethasone (0.1 or 1mg/Kg i.p.) or dexamethasone/doxofylline (0.1/0.1 mg/kg i.p.) -24, -1 and 6 hours after instillation with LPS. Vertical lines represent MEAN  $\pm$  SEM of 5-6 mice/ group.  $\delta$  p<0.0001 vs saline group, \*\*\*p<0.00001 vs LPS group, \*p< 0.001 vs LPS group, \*\*\*\*p<0.00001 vs LPS group. One way ANOVA followed by Dunnett Post Hoc.

**Figure 2. (A)** Effect of dexamethasone, doxofylline or montelukast on OVA-induced eosinophil migration into the lung. **(B)** effect of the combination dexamethasone/doxofylline and doxofylline/montelukast on OVA-induced eosinophils migration to the lung. Bronchoalveolar lavage was collected 24 h post aerosol challenge with OVA (OVA). Mice were treated with doxofylline (0.1 or 1mg/Kg i.p.), dexamethasone (0.1 or 1mg/Kg i.p.), montelukast (0.1 or 0.3 mg/kg i.p.), dexamethasone/doxofylline (0.1/0.1 mg/kg i.p.) or dexamethasone/montelukast (0.1/0.1 mg/kg i.p.) -24, -1 and 6 hours after challenge with Ovalbumin. Vertical lines represent MEAN  $\pm$  SEM of 6-11 mice (OVA experiment).  $\alpha$  p<0.001 vs sham group, \*p<0.05 vs OVA group, \*\*\*p<0.0001 vs OVA group. One way ANOVA followed by Dunnett Post Hoc.

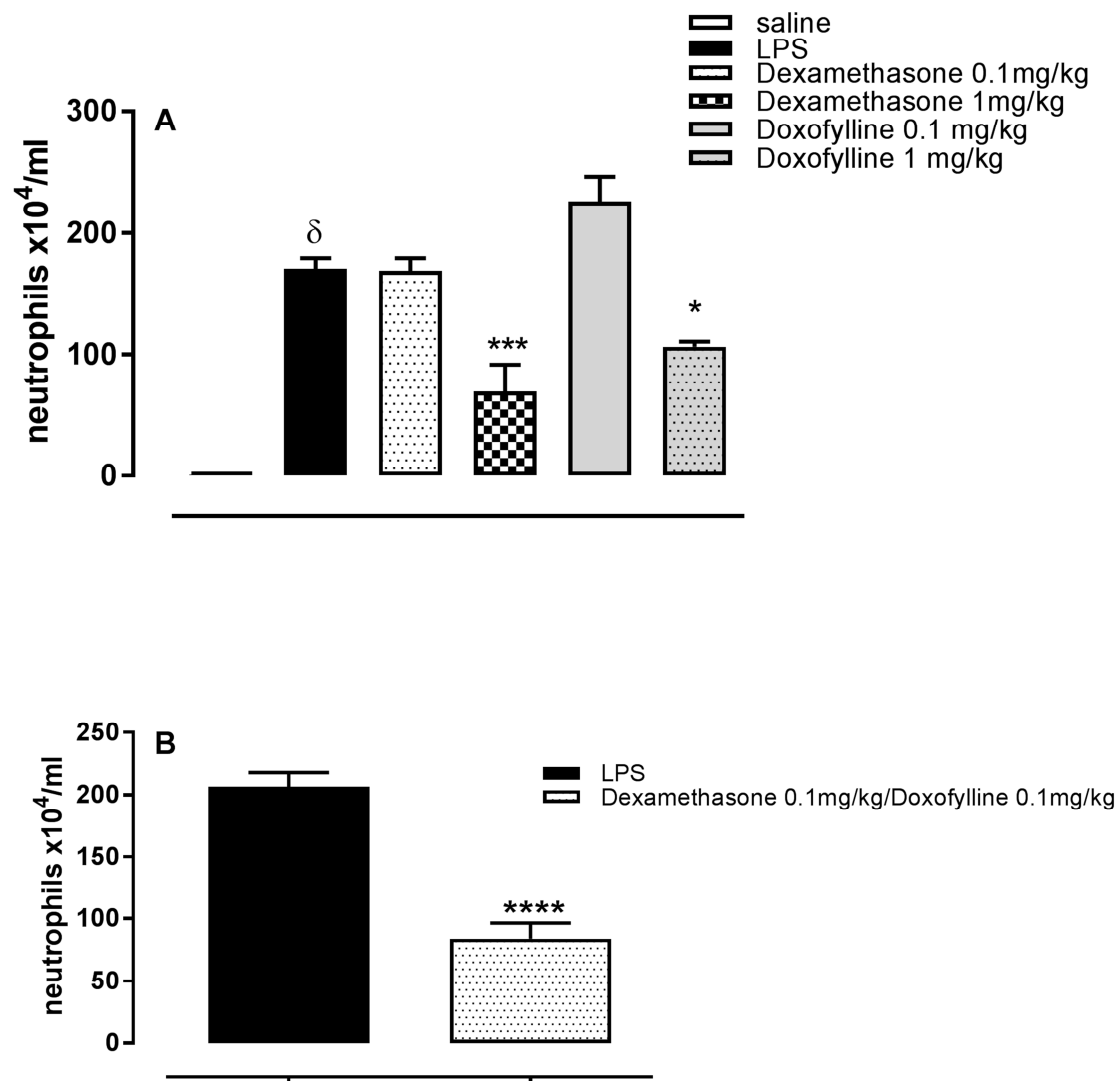


Figure 1  
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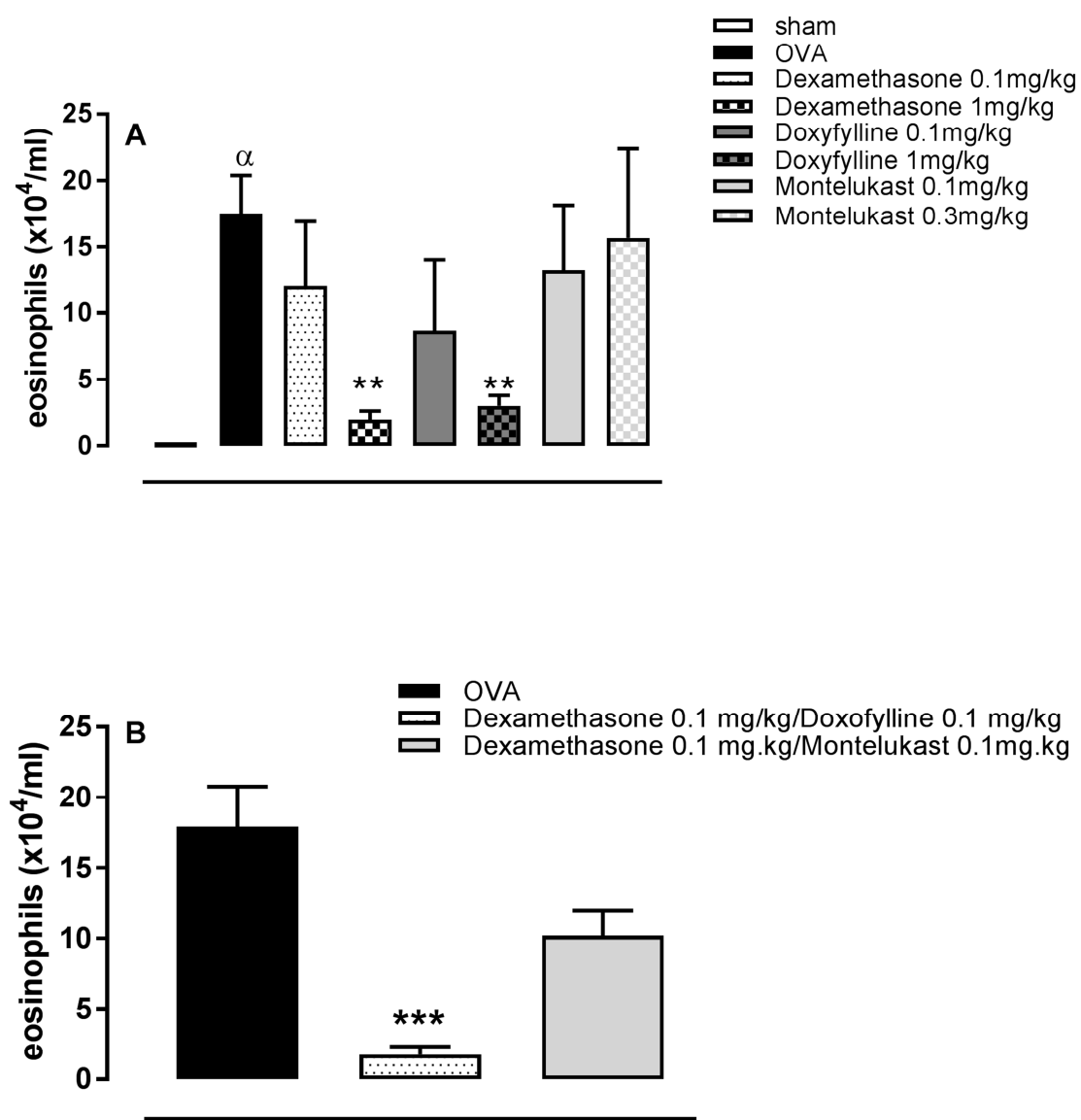


Figure 2  
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